IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

STEPHEN JOSEPH VESPER

Application No. 09/866,793

Filed: May 30, 2001

MEHTODS FOR ISOLATING AND USING FUNGAL HEMOLYSINS

Examiner: P. A. Duffy
Art Unit: 1645

APPEAL BRIEF

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Date: August 11, 2008

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REAL PARTY IN INTEREST

The real party in interest is the United States Environmental Protection Agency.

RELATED APPEALS AND INTERFERENCES

Undersigned is aware of no related Appeals or Interferences.

STATUS OF CLAIMS

Claims 1-22 have been cancelled. Claims 34-38 have been withdrawn. Claims 23-33 stand rejected. Claims 23-33 are appealed.

STATUS OF AMENDMENTS

The amendment filed June 24, 2005, will not be entered even for purposes of appeal. The Notice of Panel Decision from Pre-Appeal Brief Review determined that claims 23-33 are rejected and claims 34-38 are withdrawn.

SUMMARY OF CLAIMED SUBJECT MATTER

The present invention is directed to methods for detecting exposures to fungi in a species-specific manner. This invention is based on the discovery that fungi that were previously not known to produce hemolysins do indeed produce these proteins, which are species-specific. This is useful because different fungi are associated with different diseases. The knowledge of the specific fungus causing the disease can affect prevention methods or treatment protocols. Therefore, it is critical to be able to differentiate human or other animal exposures to a specific fungus, so that the proper medication at the proper dosage can be administered in a timely fashion.

The present invention provides that these same specific hemolysins can be used to quantify the specific fungi in environmental samples. This is useful because one could monitor a hospital, workplace, or home for the presence of problematic fungi in a timely fashion, possible preventing exposures of the people or animals in these environments.

Although it has been known for years that certain fungal pathogens such as *Candida albicans* and *Aspergillus fumigatus* produced hemolysins, the present invention describes for the first time that other fungi also produce hemolysin. The present application teaches how to use this knowledge to provide a useful product/service for the medical and environmental communities. That is, using the process of the present invention, one can determine which if any fungi are present in a sample.

The fungal hemolysin protein may be present in blood, serum, urine, saliva or other measurable body fluid of a human or other animals exposed to the fungus. If the fungus is present in the environment, such as a building, a sample from, *e.g.*, a wall or floor of the building can be taken to determine if a fungus is present in the building or other environment. The method of measurement is not critical and can include GC-MS, MALDI-tof, immunoassays such as ELISA and RIA, or the like.

Claim 23 is directed to a method for determining if an animal has been exposed to a specific hemolysin-producing fungus, which hemolysin is species-specific, by contacting a sample from an animal with labeled antibodies that bind only to the hemolysin produced by the fungus,

detecting any complex formed, and correlating presence of a complex with exposure to a fungus (specification, paragraphs 0012, 0021, 0032, 0033). Dependent claims 24-26 (paragraph 0021) define the type of sample, the label used (paragraph 0048) and the specific fungus respectively (paragraph 0035).

Claim 27 is directed to a method for determining if an animal has been exposed to a specific hemolysin-producing fungus as above wherein the fungal hemolysin used to prepare the labeled antibodies is isolated by culturing a strain of fungus and isolating hemolytically active fractions of fungal hemolysin (specification, paragraphs 0014, 0025-0033).

Claim 30 is drawn to a method for determining if a building containing hemolysin-producing fungi by assaying a sample from a building in a manner similar to assaying a sample from an animal (specification, paragraphs 0018, 0024, 0036, 0037). Dependent claim 31 is drawn to the label used (paragraph 0048) and claim 32 is drawn to the specific fungus (paragraph 0035).

Claim 33 is directed to a method for determining if an animal has been exposed to a specific hemolysin-producing fungus by detecting the presence of the hemolysin produced by the fungus in a sample from the animal, the presence of the hemolysin in the sample indicating that the animal has been exposed to the specific hemolysin-producing fungus (specification, paragraphs 0012, 0021, 0032, 0033).

GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

1. Claims 23-29 are rejected under 35 U.S.C. §103(a) as being unpatentable over Sakaguchi et al. (*Japanese Journal of Medical Mycology* **25(3)**:219-224 Abstract, 1984) in view of Harlow et al. (*Antibodies: A laboratory Manual*, cold spring Harbor Press, 1989, pages 390-393).

2. Claim 33 is rejected under 35 U.S.C. 102(b) as being anticipated by Sakaguchi et al.

3. Claims 23-29 and 33 are rejected under 35 U.S.C. 112, first paragraph as failing to comply with the written description requirement.

4. Claims 30-32 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement.

5. Claims 30-32 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the enablement requirement.

6. Claims 30-32 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

7. Claims 23-26 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. This is a new matter rejection.

In addition to the above grounds of rejection, the specification has been objected to as failing to provide proper antecedent basis for the claimed subject matter, namely, "specific hemolysin-producing fungus."

ARGUMENT

I. Claims 23 and 25-29 are not obvious over Sakaguchi et al. in view of Harlow et al. under 35 U.S.C. 103(a)

The Examiner alleges that there is no evidence in the specification that the applicants discovered that certain fungi produce hemolysins, which are specific to the particular fungus producing the hemolysin.

The present inventor discovered that hemolysin-producing fungi produce hemolysins that are specific to each fungus. The present inventor discovered that this information can be used to devise assays for these hemolysin-producing fungi based upon detection of the hemolysin that is specific to each fungus. Without the knowledge that each hemolysin producing fungus produces a hemolysin specific for that fungus, the methods of the present invention would be worthless for identifying specific fungi.

Applicant discovered that certain fungi produce hemolysins which are specific to the particular fungus producing the hemolysin. The present invention is directed to methods for assaying for specific hemolysin-producing fungi, which would not be possible if hemolysin-producing fungi did not produce species-specific hemolysin. Paragraph 0015 in the specification as filed specifically states that one object of the invention is to identify strains of fungi using an *in vitro* test. One skilled in the art would thus expect that the invention would be directed to identifying individual strains of fungi. One skilled in the art need not be told *in haec verba* that each fungus produces a unique hemolysin, because it is inherent that the hemolysins are unique if one is able to identify different strains of fungi, which is one object of the present invention. If the hemolysins were not unique to each specific fungus, one would not be able to identify different strains of fungi.

The Examiner appears to be confusing antibody specificity with hemolysin specificity. Paragraph 0024 of the specification states that by growing strains of hemolysin producing fungi *in vitro* and isolating the hemolysin, it is now possible to use the protein obtained [to produce antibodies] to identify fungi which are isolated from buildings, homes, schools and the like. If each

fungus did not produce a species-specific hemolysin, it would be impossible to identify which fungi are present, or to which fungus or fungi an animal or building has been exposed.

As best this rejection can be understood, Sakaguchi et al. has been cited for teaching that antibodies to a fungal hemolysin can be used to detect infection in an animal infected therewith. Harlow et al. are cited for disclosing methods for producing antibodies.

Sakaguchi et al. injected mice with *A. fumigatus* in order to determine how the fungus infects the body of the mouse. Sakaguchi et al. then sacrificed the mouse and detected viable fungus in the kidney and brain ten days after challenge. This information revealed the progress and mechanisms of infection.

It is not understood how the Examiner can extrapolate from a description of a study of the course of a fungal infection in mice that have been deliberately infected with a fungus and subsequent observation of infection in the kidneys, livers, internal organs of the mice, to an assay for a variety of specific fungi which does not involve taking tissue samples from the animal believed to be infected.

Sakaguchi et al knew that the mice had been infected with *A. fumigatus*, and they were trying to determine how the infection affected the organs of the mice. There is nothing in Sakaguchi et al. that even suggests that one would look to hemolysins to determine if an animal had been exposed to one of a number of hemolysin-producing fungi, because Sakaguchi et al. specifically infected the mice with *A. fumigatus*. There was no reason for Sakaguchi et al. to try to identify exposure to any other kind of fungus, because the point of the experiment was to ascertain how *A. fumigatus* infected mice.

Claims 23 and 25-29 are drawn to *A method for determining if an animal has been exposed to a specific hemolysin-producing fungus*. This preamble of the claims limits the claims to a method for determining **if** an animal has been exposed to a hemolysin-producing fungus. As the Federal Circuit said in *Manning v. Paradis*, 296 F3d 1098, 1103; 63 USPQ2d 1681 (Fed. Cir. 2002), "Just as the preamble of a count may define a limitation of the count, so too it may define the intended

purpose of the invention." In the present case, the preamble defines the invention as a method for determining if an animal has been exposed to a hemolysin-producing fungus. The Sakaguchi et al. article is irrelevant to the present invention, because Sakaguchi et al. knew that the animal had been infected with a fungus. Sakaguchi et al. had no reason to determine **if** the animal had been exposed to a hemolysin-producing fungus, because Sakaguchi et al knew that the animals tested had been exposed to *Aspergillus fumigatus*.

II. Claim 33 is not unpatentable under 35 U.S.C. 103(a) over Sakaguchi et al.

Claim 33 is drawn to a method for determining if an animal has been exposed to a specific hemolysin-producing fungus by detecting the presence of the hemolysin produced by the fungus in a sample from the animal. Sakaguchi et al. do not try to identify if an animal has been exposed to a fungus, and if so, to which fungus. Rather, Sakaguchi et al. teach a method for determining how a specific fungus infects an animal. The Examiner stated on page 10 of the Office Action mailed April 8, 2005, that the Sakaguchi et al. method would inherently do so (*i.e.*, a specific hemolysin could be used to identify each fungus). There is nothing in Sakaguchi et al. that teaches or even suggests that the assay for the hemolysin of *A. fumigatus* could be used to determine if the animal had been exposed to another fungus. Sakaguchi et al. knew that the animals had been exposed to *A. fumigatus* because the researchers had infected the animals with *A. fumigatus* and monitored the progress of the infection of the animal by determining which organs had been invaded by the fungus by detecting the presence of *A. fumigatus* in the organs. There is nothing in Sakaguchi et al. that even suggests that hemolysin-specific fungi produce individual hemolysins. All Sakaguchi et al. disclose is that one can track infection by *A. fumigatus* by monitoring the progress of the fungus through the organs of the animal.

III. Claims 23-29 and 33 comply with the written description requirement of 35 U.S.C. 112, first paragraph.

The Examiner alleges that the specification as filed lacks the conception of "species specificity" and the ability to discriminate one fungus from another based on species-specific

hemolysin. However, this is in direct contrast to her allegation that Sakaguchi et al. inherently disclose that a specific hemolysin can be used to identify a specific fungus, *i.e.*, that a hemolysin is species-specific.

The specification at paragraph 0012 states that an object of the invention is to provide a method and reagent for screening humans and other animals for exposure to hemolysin-producing fungi. Paragraph 0015 states that it is a further object of the invention to identify strains of fungi using an *in vitro* test. This *in vitro* test involves determining the presence of a hemolysin, and correlating the presence of that hemolysin to infection by the fungus that produced the hemolysin. If the hemolysins were not species-specific, it would not be possible to identify strains of fungi. If the hemolysins were not species-specific, the present application would be inoperable, as it is the specific hemolysins that make it possible to identify different species of fungi.

The present invention is directed to methods for detecting fungi that produce hemolysin in order to demonstrate exposure to these hemolysin-producing fungi. Humans do not produce normal antibodies in response to exposure to many fungi or mycelium of many fungi. Therefore, traditional immunoassay methods cannot be used to detect exposure to hemolysin-producing fungi.

The present inventor has devised a method for isolating fungal hemolysin and using the hemolysin proteins obtained to demonstrate exposure to fungi for environmental or medical evaluations. The hemolysins produced are specific to each fungus that produces a hemolysin. The fungal hemolysin protein may be present in blood, urine, saliva, or other measurable body fluid of a human or other animal infected with the fungus. The assay is conducted with antibodies, produced by conventional techniques, against the particular hemolysin protein. The assay can be any conventional immunoassay, such as ELISA, RIA, or the like.

An objective standard for determining compliance with the written description requirement is, "does the description clearly allow persons of ordinary skill in the art to recognize that the or she invented what is claimed." *In re Gosteli*, 872 F2d 1008, 1012, 10 USPQ 1614, 1618 (Fed.

Cir. 1989). For purposes of the present invention, it is assumed that one skilled in the present art is familiar with clinical assay techniques, including immunoassays, to detect antigens in a sample.

It is respectfully submitted that claims 23-29 and 33 contain subject matter that was described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor, at the time the application was filed, had possession of the claimed invention. The present inventor has described a method for isolating hemolysin from a hemolysin-producing fungus at paragraphs 25-27. The specification at paragraphs 28-33 describes how the hemolysin can be used to obtain antibodies, which then are used in an immunoassay to detect presence of the fungus. Paragraph 32 clearly states that the antibodies to fungal hemolysin can be used in a conventional immunoassay to determine if one has been exposed to strains of fungi which produce hemolysin. One skilled in the art of clinical assays is well aware of many conventional immunoassays that can be used to detect antibody-antigen reactions. There is no need to be specific with respect to the particular assay used.

IV. Claims 30-32 comply with the written description requirement of 35 U.S.C. 112, first paragraph.

The specification beginning at paragraph 36 describes how to determine if a building holds fungi which may be a problem to the inhabitants or users thereof. In this method, a strain of fungus obtained from the building is cultured and the culture filtrate applied to a plate. If the filtrate is shown to be hemolytic, the strain is problematic and may pose health risk.

It is respectfully submitted that this description of screening fungi in a building is perfectly clear to one skilled in the art and demonstrates that the inventor possessed the invention at the time of filing the present application. One skilled in the art of assays would certainly know how to obtain a representative sample from a building in order to determine if the building harbored problematic fungi. One skilled in the art would also appreciate that an analysis of the filtrate from the culture would contain hemolysins from any hemolysin-producing fungi present, and could be assayed in the same manner as samples from bodily fluids. One skilled in the art does not need to have each

step spelled out in excruciating detail, as one skilled in the art can readily ascertain how to obtain suitable samples and proceed with such an assay.

V. Claims 30-32 comply with the enablement requirement of 35 U.S.C. 112, first paragraph.

One skilled in the art of clinical assays or assaying for deleterious microorganisms in a building or other such environment, could read the present specification and readily contrive to conduct such assay without undue experimentation. One skilled in the art of these assays would know how to obtain samples from a building or other environment, and then, as described at paragraphs 25 to 32, assay for the presence of fungal hemolysins in the sample. Paragraph 36, which pertains specifically to screening fungi in a building, even discloses the culturing technique for growing the suspected hemolysin-producing fungi.

VI. Claims 30-32 point out and distinctly claim the subject matter which application regards as the invention, and thus conform to the requirements of 35 U.S.C. 112, second paragraph.

The Examiner contends that the phrase "obtaining hemolysin from the sample if hemolysin-producing fungi are present in the sample" infers that the assayer know that there is hemolysin-producing fungus in the sample. This is not the case at all. Claim 30 is directed to a method for determining **if** a building contains a hemolysin-producing fungus. One obtains the sample from the building and, **if** hemolysin-producing fungi are present in the sample, one recovers hemolysin from the sample and assays it to determine what hemolysin-producing fungi are present in the building. If one knows there are hemolysin-producing fungi in the building, there would be no reason to determine if the building contains a hemolysin-producing fungus. Likewise, if there are no hemolysin-producing fungi in the building, one could not obtain hemolysin-producing fungi from the sample.

VII. Claims 23-26 comply with the written description requirement of 35 U.S.C. 112, first paragraph.

Claims 23-26 have been amended in the preamble to recite "which hemolysin is species-specific", which the Examiner alleges is new matter. The Examiner's position is that there is no inherent or implicit showing using evidence of specificity on the part of hemolysins from different fungi or different species within the same genus. However, as the Examiner is well aware, there is no requirement that support be *in haec verba* for the claims. It is well understood by those skilled in the art reading the present specification that hemolysin-producing fungi produce hemolysins that are sufficiently specific for each fungus so that one skilled in the art can differentiate among hemolysin-specific fungi, as well as determine which, if any, hemolysin-specific fungi are present in a sample.

While it has not been recited in the specification in the same manner as claims 23-26 are worded, it is clear that one skilled in the art would appreciate that hemolysin-producing fungi produce species-specific hemolysins. Otherwise, it would be impossible to assay for specific hemolysin-producing fungi. That the hemolysin is species-specific is inherent in the hemolysin-producing fungi, or the assay of the present invention could not possibly assay for individual fungi. As the court stated, in *Technicon Instruments Corporation v. Coleman Instruments, Inc. et al.,* "By disclosing in a patent application a device that inherently performs a function, operates according to a theory, or has an advantage, a patent applicant necessarily discloses that function, theory or advantage even though he says nothing concerning it. The application may later be amended [to] recite the function, theory or advantage without introducing prohibited new matter." 255 F.Supp. 630, 150 USPQ 227 (N.D. Ill. 1966), aff'd 385 F.2d 391, 155 USPQ 369 (7th Cit. 1967).

The Court of Customs and Patent Appeals has long recognized that an invention may be described in different ways and still be the same invention. In *In re Kirchner*, the court held that compliance with section 120 does not require that the invention be described in the same way, or comply with section 112 in the same way, in both applications.

In *Kirchner* the court authorized the addition to the specification of descriptive matter concerning the use of the compounds without loss of the parent application's filing date. In *In re*

Nathan, 51 C.C.P.A. 1059, 328 F.2d 1005, 1008-09, 140 U.S.P.Q. (BNA) 601, 604 (CCPA 1964), the court held that the later-added limitation to the claims of the compound's alpha orientation was "an inherent characteristic" of the claimed subject matter, and reversed a new matter rejection. The *Nathan* court explained that "a subsequent clarification of or a change in an original disclosure does not necessarily make that original disclosure fatally defective." *Id.* at 1008, 140 USPQ at 603.

It is clear from a reading of the present application that hemolysin-producing fungi produce hemolysin that is species specific, so that individual fungi can be identified. It is respectfully submitted that the recitation of "species-specific hemolysin" is not new matter, but is an inherent property of the hemolysin produced by hemolysin producing fungi.

OBJECTIONS/REJECTIONS MAINTAINED

The specification is objected to as failing to provide proper antecedent basis for the claimed subject matter. The Examiner alleges that there is no basis for the term "specific hemolysin-producing fungus."

Paragraph 0033 of the specification as filed states,

> The present invention thus provides a method to determine if a human or other animal has been exposed to a hemolysin-producing fungus such as *Stachybotrys chartarum*. By analyzing samples from a human or other animal for antibodies to a hemolysin-producing fungus, it is now possible to determine if the human or other animal has been exposed to such a fungus.

It is respectfully submitted that paragraph 33 clearly states what is claimed herein, paragraphs 0028-0032 teaches how to prepare antibodies to the fungal hemolysin. In each instance, it is clear that each fungus produces a hemolysin that can be detected. There is nothing in the entire specification that would lead one skilled in the art that multiple fungi produced the same hemolysin, *i.e.*, that an assay for a fungal hemolysin would not reveal which fungus produced the hemolysin.

CONCLUSION

Appellants respectfully submit that the Examiner's rejections are unreasonable, that no *prima facie* case of obviousness has been established, and that the claims are fully enabled by the specification and the written description.

The prior art provides no incentives to test for a specific hemolysin-producing fungus. One skilled in the art, reading the specification as a whole, can appreciate that the inventor possessed the invention at the time of filing, and one skilled in the art can practice the present invention without undue experimentation.

The rejections should be reversed and such is respectfully prayed.

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CLAIMS APPENDIX

23. A method for determining if an animal has been exposed to a specific hemolysin-producing fungus, which hemolysin is species-specific, comprising:

a. contacting a sample from said animal with labeled antibodies which bind to the hemolysin produced by the fungus or to active fragments of the hemolysin; and

b. detecting any complex formed between the labeled antibodies and the hemolysin or active fragments thereof.

24. The method according to claim 24 wherein the sample from the animal is selected from the group consisting of blood, urine, and saliva.

25. The method according to claim 23 wherein the label is selected from the group consisting of enzyme, radioactive, chemiluminescent, and fluorescent labels.

26. The method according to claim 23 wherein the fungus is selected from the group consisting of *Stachybotyrus chartarum, Aspergillus fumigatus, Candida albicans*, and *Penicillium chrysogenum*.

27. A method for determining if an animal has been exposed to a specific hemolysin-producing fungus comprising:

a. contacting a sample from said animal with labeled antibodies which bind to a hemolysin produced by the fungus or to active fragments of the hemolysin produced by the fungus;

b. detecting any complex formed between the labeled antibody and any hemolysin or active fragments thereof in the sample;

c. wherein the fungal hemolysin used to prepare the labeled antibodies is isolated by culturing a strain of fungus, removing cells and debris from the culture to

recover supernatant, and isolating hemolytically active fractions of fungal hemolysin.

28. The method according to claim 27 wherein the label is selected from the group consisting of enzyme, radioactive, chemiluminescent, and fluorescent labels.

29. The method according to claim 27 wherein the fungus is selected from the group consisting of *Stachybotyrus chartarum, Aspergillus fumigatus, Candida albicans*, and *Penicillium chrysogenum*.

30. A method for determining if a building contains a hemolysin-producing fungus comprising:

a. obtaining a sample from the building;

b. obtaining hemolysin from the sample if hemolysin-producing fungi are present in the sample;

c. contacting the sample with labeled antibodies which bind to the fungal hemolysin or to active fragments of the fungal hemolysin; and

d. detecting any complex formed between the labeled antibodies and the fungal hemolysin or active fragments thereof.

31. The method according to claim 30 wherein the label is selected from the group consisting of enzyme, radioactive, chemiluminescent, and fluorescent labels.

32. The method according to claim 30 wherein the fungus is selected from the group consisting of *Stachybotyrus chartarum, , Candida albicans*, and *Penicillium chrysogenum*.

33. A method for determining if an animal has been exposed to a specific hemolysin-producing fungus comprising detecting the presence of the hemolysin produced by the fungus in a sample from the animal, the presence of the hemolysin in the sample indicating that the animal has been exposed to the hemolysin-producing fungus.

WITHDRAWN CLAIMS

34. A method for determining if an animal has been exposed to a specific hemolysin-producing fungus by determining if a sample from the animal contains antibodies to a hemolysin, comprising:

a. contacting a sample from said animal with labeled hemolysin from a suspected hemolysin-producing fungus; and

b. detecting any complex formed between the labeled hemolysin and antibodies to the hemolysin.

35. The method according to claim 34 wherein the sample from the animal is selected from the group consisting of blood urine, and saliva.

36. The method according to claim 34 wherein the label is selected from the group consisting of enzyme, radioactive, chemiluminescent, and fluorescent labels.

37. The method according to claim 34 wherein the fungus is selected from the group consisting of *Stachybotyrus chartartum, Candida albicans*, and *Penicillium chrysogenum*.

38. A method for determining if a building contains fungi which may be deleterious to occupants of the building comprising:

a. obtaining a strain of a fungus from the building;

b. culturing the fungus;

c. applying the culture filtrate to a plate; and

detecting the presence of hemolysin in the plate.

EVIDENCE APPENDIX

1. HARLOV et al., *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988, pp. 390-393

2. SAKAGUCHI et al., *Japanese Journal of Medical Mycology* **25(3)**:219-224, 1984 ABSTRACT ONLY

3. Declaration of Stephen J. Vesper submitted with amendment filed July 8, 2002

RELATED PROCEEDINGS APPENDIX

There are no related proceedings in connection with the subject application.

Antibodies

A LABORATORY MANUAL

Ed Harlow

Cold Spring Harbor Laboratory

David Lane

Imperial Cancer Research Fund Laboratories

Cold Spring Harbor Laboratory
1988

Antibody Binding

Once cells or tissues are fixed and permeabilized, the antibodies are added. Here, as in many other immunochemical techniques, the antibodies can be labeled directly or they can be detected by using a labeled secondary reagent that will bind specifically to the primary antibody. Both the direct and indirect detection methods are in common use, and the choice of method will depend on the experimental design. In general, direct labeling of the primary antibody will produce cleaner signals with lower background. The major disadvantage of direct detection is the time needed to purify and label each preparation of primary antibody. Another potential disadvantage to using direct detection is that the signal will not be as strong as for indirect methods, where the number of labeled molecules will be higher. The purification and labeling of antibodies are discussed in Chapters 8 and 9 (pp. 283 and 319).

For indirect detection, any reagent that will bind specifically to the primary antibody can be "tagged" and used to locate the antibody. The possible reagents include anti-immunoglobulin antibodies (p. 624), protein A or G (p. 615), or, if the first antibody is labeled with biotin, streptavidin (p. 340). The major advantage of indirect detection is that one set of labeled reagents can be used for a number of primary antibodies. Indirect methods will normally give stronger signals, but the backgrounds may be worse.

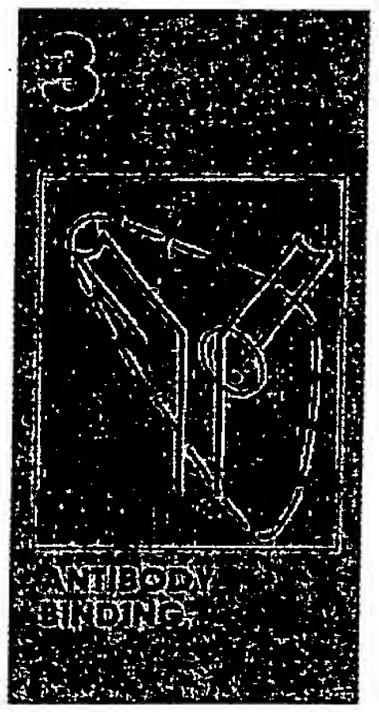
Detection reagents for cell staining can be labeled with fluorochromes, enzymes, gold, or iodine. Each of the labels has advantages and disadvantages that will vary between different experimental designs. The choice of label is discussed in the detection section below (p. 396). Methods for labeling these reagents are listed in Chapter 9.

Because the antigen in cell staining will be fixed to a solid phase, the time needed for the antibody to find the antigen will be longer than if both molecules are in solution. The incubation times can be adjusted for the experimental design, but seldom will times less than 30 min yield efficient binding. The incubation times can be lowered by increasing the concentration of the antibodies, but this will also increase the background. Usually, some compromise needs to be reached between using enough to achieve a good signal and keeping the background to an acceptable level. In all cases, the antibodies should be diluted in buffers containing high concentrations of nonspecific proteins. Proteins that are commonly used are bovine serum albumin (BSA), fetal bovine serum (FBS), nonfat dry milk, or serum from the same species as the labeled antibody.

COMMENTS ■ Controls

Specific cell staining reactions should always be compared with control reactions. To assess a staining pattern accurately, two antibodies from the same species should be compared. The best control will be a prebleed from the same animal used to prepare the specific antibodies, but nonimmune sera from the same species is acceptable in most cases. For monoclonal antibodies, the control must be from the same source as the specific antibody, i.e., supernatant versus supernatant, ascites versus ascites, or pure antibody versus pure antibody. If possible, the control antibodies should be of the same class and subclass as the specific antibody. Tissue culture supernatants from the parental myeloma are never appropriate controls, because they do not contain antibodies. Suitable control hybridoma cell lines are available from ATCC.

In addition, if using indirect detection, the secondary reagent should be tested on its own. Finally, when using enzyme-linked detection, the enzyme reaction should be done on the specimen without the addition of any antibodies. This will demonstrate the presence and location of any endogenous enzyme activities.



BINDING ANTIBODIES TO ATTACHED CELLS

Antibodies generally are applied directly to the area of the cells or tissues that is being studied.

1. Cells are fixed and washed as described on p. 384. Place coverslips, slides, or plates in a humidified chamber. Slides or coverslips can be placed in a petri dish containing a water-saturated filter. Coverslips are best placed on a layer of parafilm; this helps to stop the antibody solution from rolling off the edge of the coverslip and makes it easy to pick up the coverslips with fine forceps, as the parafilm is compressible.

2. Add the first antibody solution. All dilutions must be carried out in protein-containing solutions. For example, use PBS containing 3% BSA.

   For unlabeled primary antibodies: Monoclonal antibodies are best applied as tissue culture supernatants (specific antibody concentration of 20–50 μg/ml, use neat). Ascites fluids, purified monoclonal and polyclonal antibodies, and crude polyclonal sera should be tested at a range of dilutions aimed at producing specific antibody concentrations between 0.1–10 μg/ml. If the specific antibody concentration of the antibody sample is unknown, prepare and test 1/10, 1/100, 1/1000, and 1/10,000 dilutions of the starting material.

   For labeled primary antibodies: Primary antibodies can be labeled with enzymes, fluorochromes, or iodine as described on p. 319. They should be assayed at several dilutions in preliminary tests to determine the correct working range. Too-high concentrations will yield high backgrounds; too-low concentrations will make detection difficult. The correct concentration will depend on both the abundance of the antigen under study and the specificity of the antibody.

3. Incubate the coverslips, slides, or plates for a minimum of 30 min at room temperature in the humidified chamber. For some reactions, prolonged incubations of up to 24 hr can increase sensitivity.

4. Wash in three changes of PBS over 5 min. This buffer may be supplemented with 1% Triton X-100 or NP-40 to help with any background problems.

If the first antibody is labeled, the specimen is now ready for the detection step (p. 396).

5. Apply the labeled secondary reagent. It is essential to carry out all dilutions in a protein-containing solution such as 3% BSA/PBS or 1% immunoglobulin/PBS (prepared from the same species as the detection reagent). Useful secondary reagents include anti-immunoglobulin antibodies, protein A, or protein G (see Chapter 15). They can be labeled with enzymes, fluorochromes, gold, or iodine. Labeled secondary reagents can be purchased from several suppliers or can be prepared as described on p. 319.

   For enzyme-labeled reagents: If using a commercial preparation, test dilutions of the secondary antibodies 1/50 to 1/1000. Alkaline phosphatase-labeled reagents should be handled using Tris-buffered saline, not PBS.

   For fluorochrome-labeled reagents: If using commercial preparations, test dilutions between 1/10 to 1/300.

   For gold-labeled reagents: Wash the gold particles once in PBS. Dilute in PBS containing 1% gelatin and add to the specimen.

   For iodine-labeled reagents: Add the iodinated antibody at approximately 0.1 μg/ml. Usually, specific activities between 10 and 100 μCi/μg are used.

6. Incubate with the labeled secondary reagent for a minimum of 20 mins at room temperature in the humidified chamber. For gold-labeled reagents, observe periodically under the microscope until a satisfactory signal is obtained.

7. Wash in three changes of PBS (or Tris saline) over 5 min.

The specimen is now ready for the detection step (p. 396).

NOTES

i. When using horseradish peroxidase-labeled reagents, the buffers used for dilution and washing should not contain sodium azide.

ii. If background problems are seen, the nonspecific binding can often be inhibited by preincubating the specimen with protein. Commonly used proteins are BSA at 3%, fetal bovine serum at 10% (use fetal and not calf, as fetal bovine serum has lower amounts of IgGs), 10% dry milk, or purified antibodies (used at 1%) from the same species as the detection reagent. The blocking protein can be added to each antibody preparation and/or can be used to incubate the samples before the addition of antibody.

APPL COPY

2/7/8 (Item 1 from file: 50)
DIALOG(R)File 50:CAB Abstracts

01619059 CAB Accession Number: 851308347

A role of haemolytic toxin and its biological activity during experimental %%%Aspergillus%%% infection in mice.

Sakaguchi, O.; Yokota, K.; Ebina, K.; Ishizuka, Y.
First Dep. Hygienic Chem., Tohoku Coll. Pharm., Sendai, Miyagi 983, Japan.

Japanese Journal of Medical Mycology vol. 25 (3): p.219-224
Publication Year: 1984
ISSN: 0583-0516
4 fig., 1 tab. --
Language: Japanese Summary Language: english
Document Type: Journal article

Mice were injected intravenously with A. fumigatus at 5 x 106 and 107 viable spores. Viable %%%fungus%%% was detected in the kidney and brain 10 days after challenge. Secretion of Asp-%%%hemolysin%%% from the mycelia was observed immunohistochemically in the tissues using a technique of indirect enzyme labelled peroxidase binding IgG %%%antibody%%%. With the simultaneous administration of Asp-%%%hemolysin%%% and A. fumigatus, the toxin was shown to be a virulent factor in the infection. Pretreatment with antitoxin IgG-%%%antibody%%% exhibited a greater protective effect against A. fumigatus invasion than did IgM %%%antibody%%%. Lesions of various degrees were observed in the kidney, heart, liver and brain of mice injected with the toxin, which bound to the arterial walls in the kidney and brain. Intraperitoneal injection caused an increase in capillary permeability. The toxin showed a higher cytotoxicity to human leukocytes and guinea pig alveolar macrophages in vitro. 18 ref.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

ATTY.'S DOCKET: VESPER1

In re Application of:

S.J. VESPER

Serial No.: 09/866,793

Filed: May 30, 2001

For: METHODS FOR ISOLATING AND USING FUNGAL HEMOLYSINS

Art Unit: 1645

Examiner: SHAHNAN-SHAH

Confirmation No. 5662

Washington D.C.

DECLARATION OF STEPHEN VESPER

I, Stephen Vesper, do declare that I am an inventor of the above-identified application. In order to demonstrate that extrapolating information in the specification as filed for *Stachybotrys chartarum* to other fungi, experiments were conducted under my direction and control to demonstrate that it is easy to determine which fungi produce hemolysin, and that it is easy to obtain hemolysin from a variety of fungi.

As demonstrated by the results shown in Table 1, one skilled in the art can readily determine if a fungus exhibits hemolytic activity.

Table 1. Observations of hemolytic activity by indoor fungi. In each case, 10 µl of each conidial suspension was plated on sheep's blood agar and incubated at 37 and 23°C. The "asterisks" represent amount of growth. One asterisk is small amount of growth, two is moderate growth and three represents good growth. If there is no growth then there is no hemolysin. To ensure that the conidia were alive they were all grown at 23°C on SBA. (Only in a few cases were hemolysins produced at 23°C.)

Fungal species	EPA #	Growth Hemolysin at		Growth Hemolysin at	
		37°C	37°C.	23°C	23°C
Absidia corymbifera	133	***	No	***	No
Acremonium strictum	547	No	-	**	No
Alternaria alternata	628	*	No	**	No
Aspergillus auricomus	332	No	-	**	No
Aspergillus caespitosus	371	*	No	***	No
Aspergillus versicolor	524	*	Yes	*	No
Aspergillus candidus	329	No	-	*	No
Aspergillus carbonarius	343	*	No	**	No
Aspergillus cervinus	351	No	-	*	No
Aspergillus clavatus	350	*	No	**	No
Aspergillus flavipes	610	*	Yes	**	No
Aspergillus flavus	532	***	Yes	**	No
Aspergillus fumigatus	526	***	Yes	**	No
Aspergillus niveus	361	**	Yes	**	No
Aspergillus niger	88	***	Yes	***	Yes
Aspergillus ochraceus	426	*	Yes	**	No
Aspergillus paradoxus	373	No	-	**	No
Aspergillus parasiticus	525	***	Yes	**	No
Aspergillus puniceus	368	No	-	**	No
Aspergillus restrictus	458	No	-	*	No
Aspergillus sclerotiorum	237	*	Yes	**	No
Aspergillus sydowii	421	**	Yes	**	No
Aspergillus tamarii	607	***	No	***	No
Aspergillus torreus	231	***	Yes	**	No
Aspergillus ungulo	364	**	Yes	**	No
Aspergillus ustus	427	*	No	**	No
Aspergillus versicolor	524	*	Yes	*	No
Aspergillus wentii	608	No	-	**	Yes
Aureobasidium pullulans	701	No	-	*	No
Chaetomium globosum	396	**	No	***	No
Cladosporium cladosporioides	178	No	-	*	No
Cladosporium cladosporioides	174	No	-	*	No
Cladosporium herbarum	69	No	-	*	No
Cladosporium sphaerospermum	416	No	-	*	No
Emericella nidulans	527	***	Yes	**	Yes
Emericella variecolor	684	*	Yes	**	No

Epicoccum nigrum	335	No	-	++	No
Eurotium chevalieri	520	+	No	+	No
Memmeniella echinata	394	+	Yes	++	No
Myrothecium verrucaria	115	+++	Yes	++	No
Mucor racemosus	138	No	-	+++	No
Paecilomyces varioti	75	++	Yes	+	No
Penicillium aethiopicum	310	+	Yes	++	No
Penicillium atramentosum	601	No	-	++	No
Penicillium aurantiogriseum	441	No	-	++	No
Penicillium brevi-compactum	435	No	-	++	Yes
Penicillium canescens	437	+	Yes	+	No
Penicillium chrysogenum	467	+	Yes	+++	No
Penicillium citreonigrum	277	No	-	+	No
Penicillium citrinum	448	+	Yes	+	No
Penicillium coprophilum	600	No	-	++	No
Penicillium corylophilum	434	No	-	+	No
Penicillium crustosum	445	No	-	++	No
Penicillium decumbens	430	+	Yes	+	No
Penicillium digitatum	316	No	-	+	No
Penicillium expansum	54	No	-	++	No
Penicillium fellutanum	421	+	Yes	+	No
Penicillium glandicola	449	No	-	++	No
Penicillium griseofulvum	456	+	Yes	+	No
Penicillium implicatum	452	No		+	No
Penicillium islandicum	616	+	Yes	+	No
Penicillium italicum	59	No	-	+	No
Penicillium janthinellum	521	++	Yes	++	No
Paecilomyces lilacinus	548	+	Yes	++	No
Penicillium lividum	292	No	-	+	No
Penicillium melinii	451	No	-	+	No
Penicillium miczynskii	443	No	-	++	No
Penicillium olsonii	523	No	-	++	No
Penicillium oxalicum	497	+++	Yes	++	No
Penicillium purpurogenum	306	+	No	+	No
Penicillium raistrickii	442	No	-	++	No
Penicillium restrictum	613	+	Yes	+	No
Penicillium roqueforti	312	No	-	++	No
Penicillium sclerotiorum	453	No	-	++	No
Penicillium simplicissimum	603	+	Yes	++	No
Penicillium spinulosum	447	No	-	++	No
Penicillium variabile	322	No	-	+	No
Penicillium verrucosum	440	No	-	++	No
Penicillium waksmanii	432	No	-	++	No
Rhizopus stolonifer	58	No	-	+++	No

Scopulariopsis brevicaulis	408	**	Yes	*-	No
Scopulariopsis brumptii	409	-	No	*	No
Scopulariopsis chartarum	176	No	-	*	No
Stachybotrys chartarum	388	**	Yes	**	No
Trichoderma asperellum	538	**	No	**	No
Trichoderma harzianum	147	*	No	***	No
Trichoderma longibrachiatum	519	***	Yes	***	No
Trichoderma viride	405	No	-	***	No
Ulocladium atrum	629	*	Yes	**	No
Ulocladium botrytis	630	*	Yes	**	No
Ulocladium chartarum	631	*	No	**	No
Wallemia sebi	419	No	-	*	No

As shown in Table 1, the screening for the production of a hemolysin is simply done by placing conidia (spores) of a given fungus on 5% sheep's blood agar (SBA) (Becton Dickinson, Sparks, MD) and incubating the plates at 37°C. In a week, 92 fungi were screened for hemolysin production by looking for typical darkening then clearing of the red blood cells around the colony. This is simple, inexpensive and fast. Then all one has to do is take a fungus that produce an hemolysin and grow in TSB broth as the patent teaches. Then collect the supernatant. This is important because the homogenate (like used for asp-hemolysin) introduces a huge number of other fungal proteins whereas the supernatant is relatively free of most other fungal proteins and makes the whole purification much easier and more meaningful since the secreted form of the hemolysin is the active form. Then the hemolysin is purified using standard protein purification procedure described in the patent:

Step 1- size fractionation centrifugation; Step 2- ion chromatography; Step 3- gel filtration. Obviously there is slight variations in buffers, salt concentration, gel matrix etc. but these are accepted variations that any protein biochemist expects.

To demonstrate how easy it is to isolate other fungal hemolysins using the specified directives in the patent application, I isolated the hemolysin from *Penicillium chrsogenum* using the same steps which are growth of culture in tryptic soy broth (TSB), size fractionation centrifugation of supernatant, ion chromatography, and gel filtration.

The indoor fungus *Penicillium chrysogenum* was grown on pieces of dry wall, as described (19) and the conidia recovered. Approximately 1 x 10^5 conidia were added to 500 ml of TSB. The cultures were incubated at 23°C for 48h on an incubator shaker at 100 rpm. Then the cultures were transferred to an incubator shaker and culture for 72 h at 34°C. The fungal mass was then removed by filtering through a Whatman 541 filter paper in a Buchner funnel. The recovered filtrate was centrifuged in a Millipore Centricon plus 30 filter apparatus with a MW cut-off of 30-kDa (Millipore, Bedford, MA) following the manufacturer's instructions. The concentrate was then subjected to ion exchange

chromatography. DEAE-cellulose (Sigma, St. Louis, MO) was hydrated in 20 mM Tris-HCl pH 8.0 for 1 hr and then poured into a column giving a final bed of 3 x 0.5 cm. Then 0.5 ml of the concentrate was introduced on the top of the column. The bed was eluted with 5 ml of the 20 mM Tris-HCl buffer and five drop fractions were collected throughout the elution and then 10 µl of each fraction was plated on sheep's blood agar (SBA) (Becton Dickinson, Sparks, MD) and hemolysis noted.

Then carboxy methyl cellulose (Sigma, St. Louis, MO) was hydrated in 20 mM Tris-HCl pH 8.0 for 24 h and then poured into a column giving a final bed of 3 x 0.5 cm. Then the five active fractions from the DEAE-cellulose ion chromatography were introduced on the top of the column. The bed was eluted with 5 ml of the 20 mM Tris-HCl buffer and five drop fractions were collected throughout the elution and then 10 µl of each fraction was plated on sheep's blood agar (SBA) (Becton Dickinson, Sparks, MD) and hemolysis noted.

The five hemolytically active fractions from the second ion exchange chromatography were then subjected to gel filtration using Sephadex G 200 (Pharmacia, Piscataway, NJ) hydrated for 72 h in the running buffer containing 0.2 M sodium azide and poured into a chromatography column to give a final bed 0.25 by

24 cm. Five drop fractions were collected at 1.5 ml per h using a fraction collector (ISCO, Lincoln, NE). Then 10 µl of each fraction was plated on SBA and incubated at 37°C and hemolysis noted at 24 h. The five hemolytically active fractions from the first gel filtration were then subjected to gel filtration using Sephadex G 100-50 (Sigma, St. Louis, MO) hydrated for 5 days in the running buffer containing 0.4 M sodium azide, 5 mM EDTA, and 1 mM PMSF and poured into a chromatography column to give a final bed 0.5 x 14 cm. Five drop fractions were collected at 1.5 ml per h using a fraction collector (ISCO, Lincoln, NE). Then 10 µl of each fraction was plated on SBA and incubated at 37°C and hemolysis noted at 48 h. The five most hemolytically active fractions from this second gel filtration were combined and then desalted twice using the D-Salt™ Polyacrylamide 6000 desalting column (Pierce, Rockford, IL.). The final desalted solution was frozen at -80°C and lyophilized using a Spin Vac (Savant Instruments, Farmingdale, NY) resulting in a lyophilized pellet.

Electrophoresis analysis

Native protein electrophoresis and SDS-PAGE were performed using the Bio-Rad Laboratories Mini-Protean™ 3 Cell and precast 4-15% Tris-HCL gels (BioRad, Hercules, CA), as per the manufacturer's instructions.

Gels were stained with silver using the Bio-Rad Silver Stain Plus™ kit by following the manufacturer's instructions (Bio-Rad, Hercules, CA).

To determine if the chrysolysin was active, a purified preparation was divided in half and run in two separate wells in a precast 4-15% Tris-HCl gel. After native protein electrophoresis, half of the gel was stained with silver, as described above, and the other half was placed on an SBA plate and incubated at 37°C for 48 h and hemolysis process photographed.

Figure 1 shows the active purified hemolysin band from *P. chroysgenum*. All of the steps described in the patent were used with modifications that any protein chemist would anticipate.

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 31 of the United States Code and that such willful false statements may jeopardize the

In re Appl. No. 09/866,793
Confirmation No. 5682

validity of the application or any patent issued thereon:

Stephen Vesper 7-5-02

G:\nnie\spivesper1\pro\july 02 declaration

Figure 1. Purified hemoysin, chrysolysin, produced by *Penicillium chrysogenum*. Left panel shows purified band of chrysolysin in "native" gel. Center panel shows appearance of sheep's blood agar (SBA) after exposure to purified chrysolysin in "native" gel for 24 h and the right panel shows appearance of SBA after 48 h exposure (SBA incubated at 37°C).